REMARKS

Applicant has discovered that DNA molecules encoding calcium-dependent protein kinase polypeptides having a protein kinase domain activate stress signaling in plants. Expression of such isolated genes in plants leads to the production of genetically-engineered plants having increased tolerance to environmental stress. The invention therefore has significant implications for increasing yields and improving the quality of plant products.

Office Action

Claims 1-7, 24-26, 36-39, and 41-46 stand rejected under 35 U.S.C. § 112, first paragraph. Claims 36-46 stand rejected under 35 U.S.C. § 102(b). Each of these rejections is addressed as follows.

Amendments

To expedite prosecution, the claims have been amended to focus on methods for protecting a plant against an environmental stress that involve expressing a DNA encoding a calcium-dependent (CDPK) polypeptide that includes a protein kinase domain, and DNAs encoding a calcium-dependent (CDPK) polypeptide consisting essentially of the PK domain. This amendment finds support in the present specification, for example, generally at page 1 (line 20) – page 2 (line 10), and specifically at page 12 (line 17) - 13 (line 6).

For the record, applicant does not agree with the present rejections and reserves the right to pursue the canceled subject matter in this or a related continuing application.

Rejections Under 35 U.S.C. § 112, first paragraph

Claims 1-7, 24-26, 36-39, and 41-46 stand rejected, under 35 U.S.C. § 112, first paragraph on the basis that the disclosure in applicant's specification is (1) not

commensurate in scope with the claimed invention and (2) fails to provide a written description of the claimed invention. For the following reasons, each of these rejections is respectfully traversed.

Enablement

Claims 1-7, 24-26, and 36-46 stand rejected under § 112, first paragraph based on the assertion that the teaching of applicant's specification is not commensurate in scope with the present claims. The rejection essentially turns on the assertion that isolating PK domain polypeptides and determining whether such polypeptides protect a plant against an environmental stress requires undue experimentation. For the following reasons, applicant respectfully traverses this basis of this rejection.

By the present amendment, applicant has focused the claimed invention, in general, on methods for protecting a plant against an environmental stress involving the expression of a calcium-dependent protein kinase (CDPK) polypeptide that includes a protein kinase (PK) domain. Such claims are clearly enabled by applicant's specification.

With respect to isolating additional genes encoding CDPK polypeptides having PK-domains, applicant notes that the specification, for example, at pages 16-20, and as depicted in Figs. 3B and 3C, provides clear guidance, using specific examples, that may be utilized for identifying and isolating a variety of DNA sequences encoding CDPK polypeptides having PK domains, from a variety of sources. In addition, the specification at pages 22-24, under the heading "Isolation of Regulators of the Stress Signal Transduction Response," provides general guidance on the routine methods known at the time the application was filed for identifying and characterizing the gene sequences required by the claims. Such standard methods described in the specification include: (1) the design and utilization of oligonucleotides and degenerate probes for cloning, (2) hybridization cloning methodologies, (3) library screening procedures, and (4) PCR-based amplification cloning strategies. Together these methods, alone or in combination,

are effective for isolating and cloning desired DNAs encoding CDPK polypeptides useful in the invention.

As evidence on this point, applicant directs the Examiner's attention to the attached publication by Saijo *et al.* (*Biochimica et Biophysica Acta* 1350: 109-114, 1997; provided as Exhibit A) entitled "cDNA cloning and prokaryotic expression of maize calcium-dependent protein kinases," on which the inventor, Dr. Sheen, is a co-author. In this publication, two maize CDPKs were cloned using degenerate oligonucleotide primers corresponding to conserved regions of the calcium-dependent protein kinase (CDPK) family. Given such an example, there is absolutely no reason to believe that applicant's specification does not enable the isolation and identification of additional CDPK genes from any plant without undue experimentation. Indeed, these approaches require only standard application of routine molecular methods. Accordingly, as noted in previous correspondence, there is no basis for concluding that one skilled in the art, equipped with applicants' sequences (as well as those already known in the literature) and standard methods known in the art, would not be able to isolate a reasonable number of nucleic acid sequences which encode CDPK genes useful in practicing the methods of the present claims.

In addition, on this issue, applicant notes that determining whether a gene encoding a calcium-dependent protein kinase (CDPK) polypeptide that includes a PK domain provides protection against an environmental stress is easily accomplished, in a straightforward fashion, simply by overexpressing the gene in a transgenic plant, exactly as taught in the specification at page 24-36.

To substantiate this point, applicant directs the Examiner's attention to the publication of Saijo *et al.* (*Plant Journal* 23: 319-327, 2000; provided as Exhibit B) entitled "Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants." Here applicant notes that Saijo cloned a rice CDPK gene, called OsCDPK7, using the maize cDNA designated ZmCDPK7 identified

in Saijo *et al.* (*Biochimica et Biophysica Acta* 1350: 109-114, 1997; provided as Exhibit A). This result provides still further evidence that CDPK genes are readily isolated and identified using standard methods that were known when the application was filed. Moreover, applicant points out that, by overexpressing the rice CDPK gene in transgenic rice plants, Saijo showed that this the CDPK gene was a positive regulator of cold and salt/drought stresses, exactly as taught in applicant's specification, for example, at page 13 (lines 3-6). These results strongly corroborate applicant's claim that additional gene sequences may be isolated from a variety of sources using standard techniques that were known in the art and that were useful for practicing the methods of the claimed invention. Furthermore, contrary to the conclusions made in the Office Action that applicant's teaching does not predict "resistance to a large number of diverse stresses," the teachings of applicant's disclosure has also been shown to be effective not only for drought (see Sheen Declaration of April 14, 2000), but also for cold and salt stresses as well.

These results confirm the specification's teaching that expression of a CDPK gene that encodes a protein kinase domain promotes protection against a number of environmental stresses (for example, drought, heat, salt, cold, etc.). Accordingly, following the specification's teachings, the skilled worker can simply express a CDPK gene in a plant and then challenge the plant with an environmental stress to evaluate the plant's tolerance to the stress. Plants having increased tolerance are easily distinguished from non-tolerant plants, and the screening of such plants having the desired tolerance to an environmental stress is readily accomplished. Such a single-step screening approach does not constitute undue experimentation as is exemplified by Saijo *et al.* (Exhibit B). Applicant therefore respectfully requests that the Examiner reconsider and withdrawn the section 112 rejection, finding that applicant's disclosure enables the full scope the claims as presently amended.

Written Description

Claims 1-7, 24-26, 36-39, and 41-46 stand rejected, under 35 U.S.C. § 112, first paragraph, as containing subject matter that was not described in the specification in such a way as to convey to one skilled in the art that the inventor had possession of the claimed invention. Applicant respectfully traverses this basis of the rejection.

With respect to the existence of additional CDPK polypeptides falling within the scope of the present claims, applicant again directs the Examiner's attention to the Saijo et al. publication that provides compelling evidence for the existence of CDPK genes that confer tolerance to multiple environmental stresses. Given such proof, there is no reason for the Office to doubt the existence of additional polypeptides that are useful in the methods of the claimed invention.

Turning to the issue that applicants have not described a representative number of species of the claimed genus, applicants first point out that "[r]epresentative examples are not required by the statute and are not an end in themselves." *In re Robins,* 429 F.2d 452, 457, 166 U.S.P.Q. 552, 555 (C.C.P.A. 1970). Rather, applicants' specification "must 'convey clearly' to those skilled in the art to whom it is addressed ... the information that [the inventor] has invented the specific subject matter later claimed." *Martin v. Mayer,* 853 F.2d 500, 505 ,3 U.S.P.Q.2d 1333, 1337 (Fed. Cir. 1987).

As noted in previous correspondence, applicants have plainly met these standards. The claimed invention encompasses methods for protecting plants against an environmental stress involving the expression of DNA molecules encoding CDPK polypeptides that include a protein kinase (PK) domain (claims 1-7); plants (and seeds and cells thereof) including DNA molecules encoding CDPK polypeptides that include a PK domain (claims 24-26); and substantially pure DNAs encoding CDPK polypeptides consisting essentially of a PK domain, where the expression of such polypeptides

increases the level of tolerance to an environmental stress in a transgenic plant and cells including such DNA molecules (claims 36-46).

Applicant's specification explicitly describes to the skilled worker what is claimed, that is, (i) methods for producing plants that are tolerant to an environmental stress (e.g., drought, salt, heat, or cold), (ii) genes encoding CDPK polypeptides encoding PK domains, and (iii) methods for isolating and identifying DNAs useful for practicing the claimed invention. For example, the inventor explicitly describes methods for producing stress tolerant plants at pages 10-11 and 36-37 of the specification. The inventor also describes, for example, at pages 16-22, not only a stress-signaling expression pathway, but also expression constructs useful for generating plants having tolerance to an environmental stress.

Together, this description provides one skilled in the art with exemplary CDPK genetic constructs, as well as methods of using CDPK protein kinase domains to engineer plants that are tolerant to an environmental stress. Clearly, the inventor was in possession of the claimed invention at the time the application was filed and provided a written description that readily enables the skilled worker to identify plants and genes falling within the claimed subject matter. Applicant's specification therefore satisfies the written description requirement of § 112. This rejection may be withdrawn.

For the all of the above-mentioned reasons, applicants respectfully request withdrawal of the § 112, first paragraph rejection.

Rejections Under 35 U.S.C. § 102(b)

Claims 36-46 were rejected under 35 U.S.C. § 102(b) as anticipated by Urao *et al.* (*Mol. Gen. Genet.* 244: 331-340, 1994). For the following reasons, applicant respectfully disagrees.

Anticipation under section 102 can be found only if a reference shows exactly what is claimed. Urao does not meet this test.

Claim 36, as amended, and claims 37- 46, which refer directly or indirectly to claim 36, are now drawn to a DNA molecule that encodes a CDPK polypeptide consisting essentially of a PK domain, as well as to cells that include such DNA molecules. Urao *et al.* discloses full-length cDNA molecules encoding full-length CDPK polypeptides. Nowhere does Urao describe a DNA molecule encoding a PK domain consisting essentially of itself. Since Urao does not identically describe the claimed subject matter, it cannot anticipate applicant's claims directed to DNA molecules encoding polypeptides consisting essentially of PK domains or cells that include such DNAs. Applicant respectfully requests reconsideration and withdrawal of the § 102(b) rejection.

CONCLUSION

Applicant submits that the claims are now in condition for allowance, and such action is respectfully requested. Enclosed is a petition to extend the period for replying for three months, to and including February 8, 2001.

If there are any charges, or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: 8 February 2001

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EXHIBIT A

FEB.7

ELSEVIER

Biochimica et Biophysica Acta 1350 (1997) 109–114

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Short sequence-paper

cDNA cloning and prokaryotic expression of maize calcium-dependent protein kinases [1]

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Abstract

Using degenerate oligonucleotide primers corresponding to conserved regions of the calcium-dependent protein kinase (CDPK) family, we carried out a polymerase chain reaction and obtained four distinct partial-length cDNAs from a maize leaf library. We then used these clones as probes for conventional screening and isolated 19 longer clones from another cDNA library of maize seedlings. These clones were classified into four groups based on their DNA cross-hybridization. Two full-length cDNAs, designated as ZmCDPK9 and ZmCDPK7, were sequenced and characterized. The predicted protein of each clone was a typical CDPK with eleven canonical subdomains of protein kinases, and four EF-hand calcium-binding motifs in its N-terminal and C-terminal halves, respectively. The catalytic and regulatory domains were linked by a well-conserved junction domain. The N-terminus of the protein also contained a consensus sequence for an N-myristoylation signal. Northern blot analysis showed that the transcription level of each gene was higher in roots and etiolated leaves than in green leaves. To confirm the calcium dependency of the maize enzymes, the entire coding region of ZmCDPK9 was subcloned into an expression vector so that it was in frame with the vector-encoded peptide tags. A cell-free extract of *Escherichia coli* transformed with the recombinant plasmid exhibited calcium-dependent phosphorylation activity, using casein as a substrate.

Keywords: Protein kinase, calcium-dependent; Calmodulin-like domain; EF-hand motif; Intracellular calcium signaling; (Maize)

In higher plants, as well as in animals and microorganisms, calcium seems to play a pivotal role as a second messenger by regulating many aspects of cellular signaling [1]. One of the possible mechanisms by which calcium acts in plants is via calcium-dependent protein kinases (CDPKs) [2]. CDPKs contain a calcium-binding regulatory domain similar to calmodulin in their C-termini. Despite the potential importance of CDPKs, little is known about their physiological functions. The only description of a complete CDPK cDNA isolated so far from maiz is that of a pollen-specific enzyme [3]. Since we have been searching for CDPKs which are involved in the regulatory phosphorylation of maize phosphoenolpyruvate carboxylase [4], we started to systemati-

Abbreviations: CDPK, calcium-dependent protein kinase or calmodulin-like domain protein kinase; PCR, polymerase chain reaction.

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[1] The nucleotide sequence data reported in this paper have been submitted to the DDBJ/EMBL/GenBank databases under the accession numbers, D85039 (ZmCDPK9), D87042(ZmCDPK7), and D87043-D87046(PCR products).

PII S0167-4781(96)00207-2

cally characterize CDPKs in maize. Here, we report the isolation and characterization of novel cDNA clones encoding maize CDPKs.

Degenerate oligonucleotide primers were designed for two conserved regions in the catalytic domain of previously cloned CDPKs [3,5–9]. They were 5'-GACCT(G/C)AAGCC(G/T/C)GAGAA-3' and 5'-GCGAATTCGGCCCA(G/A)AA(G/A/T/C)GG-(G/A/T/C)GG-3', corresponding to the sense sequence of DLKPEN and the antisense sequence of PPFWAE, respectively. The former motif is highly conserved in subdomain VIb of serine/threonine kinases, while the latter motif is rather specific to subdomain X of CDPKs (see Fig. 2). After reverse transcription of mRNA from maize (*Zea mays* FR9^{cms} × FR37) green leaves, a polymerase chain reaction (PCR) was performed for 40 cycles (94°C for 30 s, 37°C for 3 min, and 72°C for 30 s) with 20 pM each of the primers [10]. The amplified products (243-263 bp in size) were digested with *Eco*RI, and then subcloned between the *Eco*RI and *Sma*I sites of pBluescript (Stratagene), four putative CDPK clones being thus found (accession numbers, D87043-D87046).

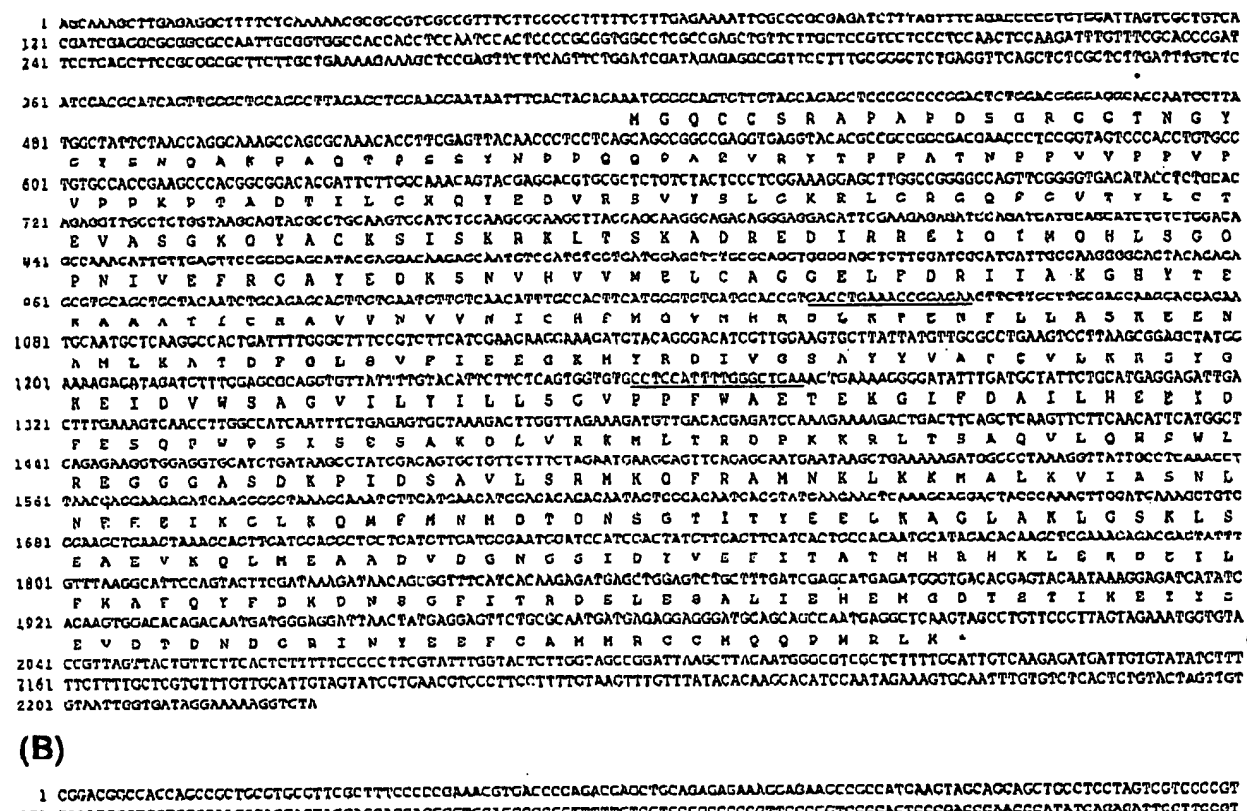
Maize (*Zea mays* H84) seedlings were grown for 7 days at 25°C under a 16-h light/8-h darkness photoperiod. Poly(A)-rich RNA of above-ground portions of the seedlings was used to construct an oligo(dT)-primed cDNA library. Double-stranded cDNA was prepared with a cDNA synthesis kit (Pharmacia), ligated with *Eco*RI/*Not*I adaptors at both ends, and then inserted into the λgt10 vector. Recombinant phages in the resulting maize cDNA library were plated with *Escherichia coli* NM514 and then blotted onto Hybond-N$^+$ membranes (Amersham). Equal amounts of the above-described four putative cDNAs were mixed, and radiolabeled with a Megaprime kit (Amersham) and [α-^{32}P]dCTP. Hybridization and washing were performed as described [11].

Nineteen positive clones were isolated out of 8×10^5 plaques. They were tentatively classified into four distinct groups based on their cross-hybridization. The first and largest group comprised nine clones. The longest cDNA insert (2.3 kb in size) among them was cut out with *Eco*RI, ligated with pBluescript, and then further analyzed. Fig. 1A shows the nucleotide and deduced amino acid sequences of the cDNA clone, ZmCDPK9. An open reading frame for a 531 amino acid polypeptide was found. The predicted molecular mass was 59.4 kDa. Exactly the same sequence as that of one of the four probes (accession no. D87043) was found in ZmCDPK9. However, the probe contained an extra segment of 20 bp. The second group comprised seven clones. Th longest cDNA insert (2.2 kb in size) among them was cut out with *Not*I, subcloned into pBluescript, and then sequenced. The clone, designated as ZmCDPK7, has an open reading frame for a 554 amino acid polypeptide, the calculated molecular mass of which is 61.1 kDa (Fig. 1B). The sequence of ZmCDPK7 differed from the corresponding probe (accession no. D87046) by two nucleotides, resulting in one amino acid substitution. These discrepancies are presumed to result from PCR errors or the difference of cultivaters. Characterization of the other two groups is now in progress.

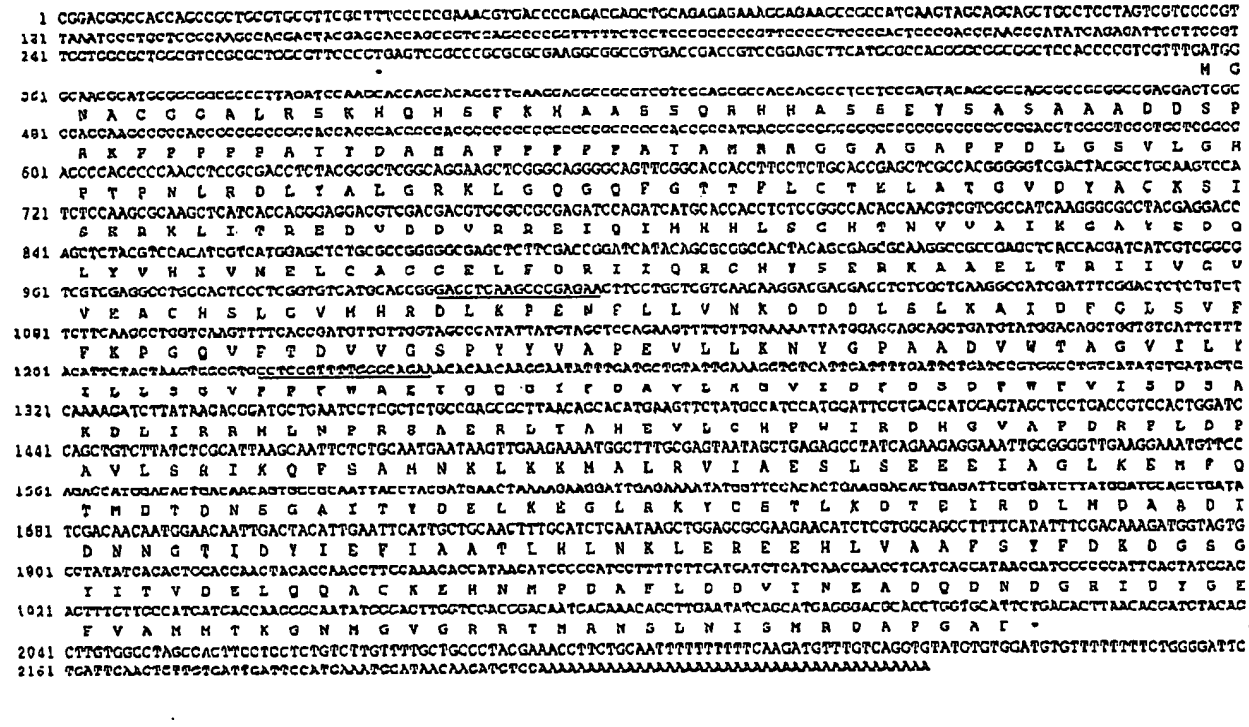
Fig. 2 shows alignment of the amino acid sequences of ZmCDPK9, ZmCDPK7 and other reported CDPKs from maize and rice. ZmCDPK9 had a kinase domain with eleven characteristic subdomains, in its N-terminal half. In the C-terminal half, on the other hand, it had a calmodulin-like domain with four EF-hand calcium binding motifs. A highly conserved junction domain was also found between the kinase and calmodulin-like domains. Thus, ZmCDPK9 encodes a typical CDPK. Similarly, ZmCDPK7 encodes another CDPK. It is noteworthy that both ZmCDPK9 and ZmCDPK7 contained N-terminal amino acid sequences (*MGQCCS* and *MGNACG*, respectively) that fulfill the requirements for a consensus sequence for N-myristoylation [12]. Therefore, the association of the enzymes with the plasma membrane was suggested. A database search revealed a maize CDPK

Fig. 1. Nucleotide and deduced amino acid sequences of ZmCDPK9 (A) and ZmCDPK7(B). Nucleotide positions (beginning at the 5' end) are indicated on the left, and amino acid positions (numbered from the putative translation initiation codon) are indicated on the right. Stop codons at the ends of the open reading frame are denoted by asterisks. Sequences corresponding to the two PCR primers are underlined.

(A)



(B)



clone, MZECDPK2 (accession no. U28376), which is very similar to ZmCDPK9 (96% identical over 496 amino acids). However, the former does not contain the myristoylation motif, suggesting that the intracellular localization of its protein product is different from that of the latter. It is also noteworthy that the clustered amino acids in the N-terminal portion of the latter differed significantly from those of the former. Another database search did not detect highly homologous maize clones to ZmCDPK7.

The ZmCDPK9 mRNA, 2.3 kb in size, was predominantly expressed in roots (Fig. 3A). In addition, its level was much higher in etiolated leaves than in green ones (Fig. 3A). The ZmCDPK7 mRNA, 2.3 kb in size, was expressed in a similar manner (Fig. 3B). It seems likely that the expression of these transcripts is repressed by light. In this regard, it is noticeable that CDPKs have been partially purified from etiolated coleoptiles and enclosed leaf rolls [13]. At present, however, it remains to be investigated if mRNA expression reflects protein levels in tissue distribution.

To examine the enzyme activity of ZmCDPK9, we cut out the entire cDNA with *Not*I, inserted it into

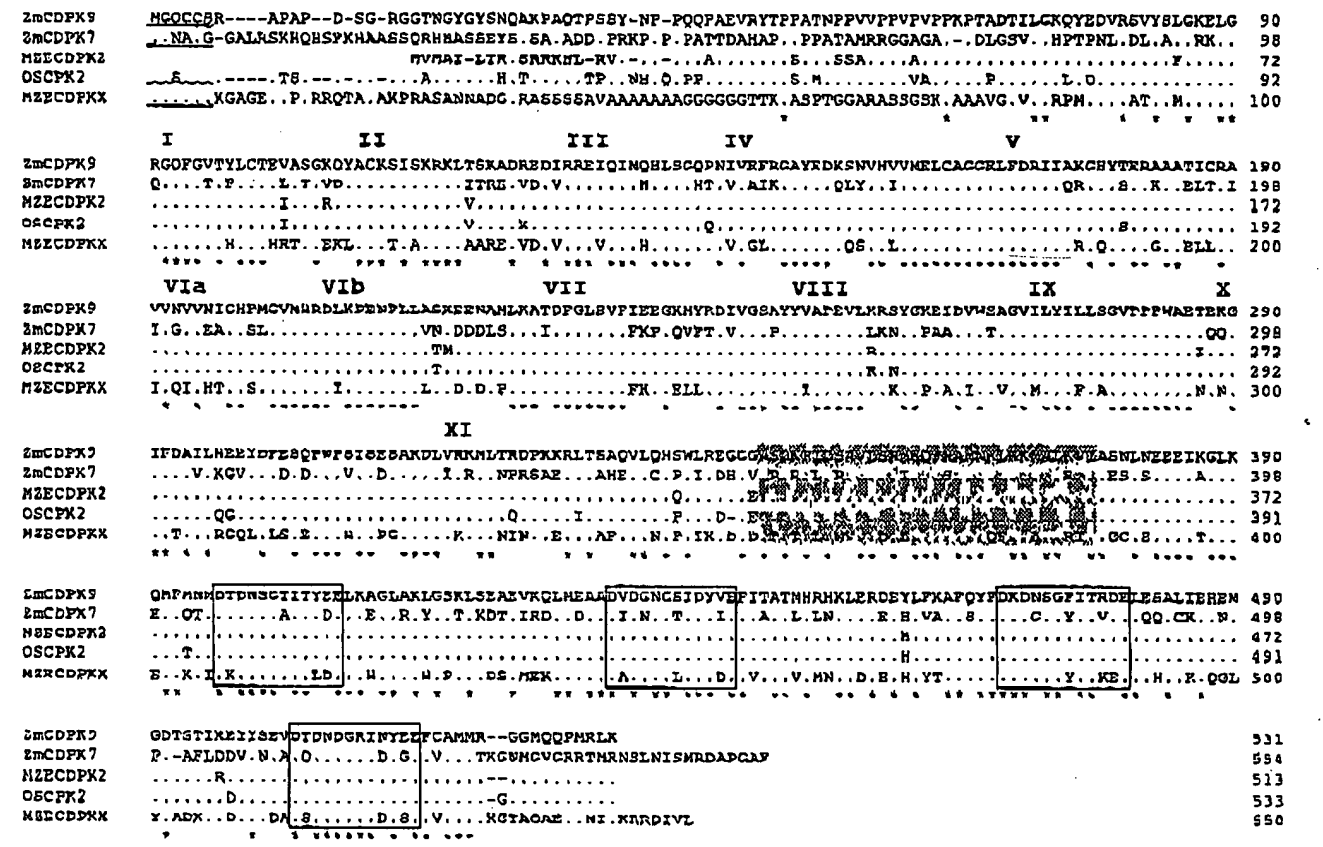


Fig. 2. Comparison of the deduced amino acid sequences of ZmCDPK9, ZmCDPK7, maize CDPKs (MZECDPKX [3] and MZECDPK2 (accession no. U28376)), and a rice CDPK (OsCPK2 [9]). Numbers indicate amino acid residues in the sequences. Dots indicate residues identical to the corresponding ones in the ZmCDPK9 sequence. Asterisks indicate residues identical throughout. Dashes indicate gaps introduced to maximize the alignment. Roman numerals indicate the eleven canonical subdomains of protein kinases identified by Hanks and Hunter [19]. The junction domain is shaded. The consensus N-myristoylation signal is underlined, and the four Ca^{2+}-binding EF-hand motifs are boxed.

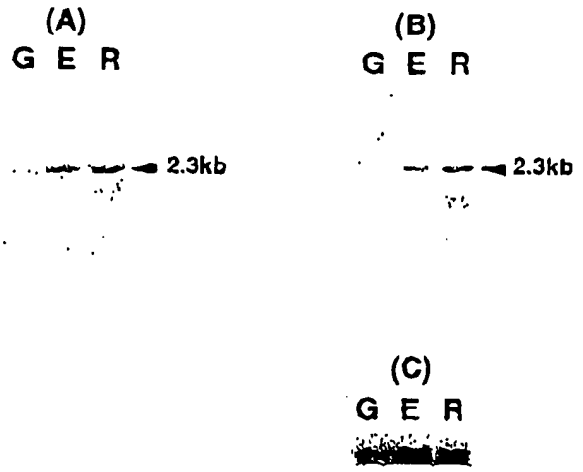


Fig. 3. Northern blot analyses of the ZmCDPK9 (A) and ZmCDPK7 (B) transcripts. Each lane contained 30 μg of total RNA isolated from different tissues of 10-day-old maize seedlings: green leaves (lane G), etiolated leaves (E), and roots (R). The RNAs were subjected to electrophoresis in a denaturing 1.2% agarose gel, and then transferred to Hybond-N$^+$ membranes. The membranes were probed with the ^{32}P-labeled 0.2 kb *Hin*dIII fragment containing the 3′-noncoding region of ZmCDPK9 or a 0.2 kb PCR fragment corresponding to nucleotides 2020-2205 of ZmCDPK7. To confirm the integrity of each RNA preparation, one of the membranes was reprobed with a cDNA for *Glycine max* ubiquitin [20] (C). The size of the detected band was estimated using a poly(A)-tailed RNA ladder (Gibco BRL) as molecular size markers.

the *Not*I site of pET32a (Novagen), and then removed 5′-noncoding region as follows. Two oligonucleotides, 5′-GACTAGAGCCATGGGGCAGTGTTG-3′ and 5′-TGTACAAAATAACACCTGCGCTCC-3′, were synthesized. The former was specific for the N-terminus of ZmCDPK9 open reading frame and contained an artificial *Nco*I site to facilitate the construction. The latter, on the other hand, was specific for the sequence corresponding to nucleotides 1218-1241 which is downstream of a natural AflII site. The PCR was carried out with the ZmCDPK9 plasmid as a template. The resulting product was digested with *Nco*I and AflII, and then cloned between the *Nco*I and AflII sites of the pET construct. Partial sequencing of the expression plasmid, designated as pETPK9, showed a correct frame for a chimeric protein of the Trx · tag, His · tag and S · tag (160 amino acids) and the entire protein kinase (531 amino acids). *E. coli* BL21 cells transformed with

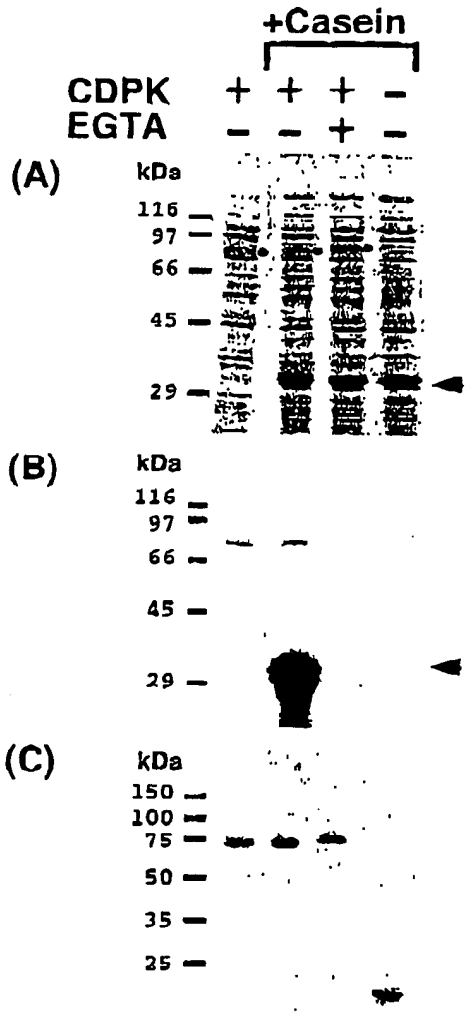


Fig. 4. Prokaryotic expression of the phosphorylation activity of ZmCDPK9 by the recombinant plasmid, pETPK9. Cell-free extracts of *E. coli* transformed with pETPK9 (CDPK+) or pET32a vector (CDPK−) were incubated with [γ-^{32}P]ATP at 25°C for 30 min with (+) or without (−) 1 mM EGTA as described in the text. The reaction products were divided into two portions and then subjected to 10% SDS-PAGE. After the electrophoresis, one gel was stained with Coomassie Blue (A) and then subjected to a Bio-imaging analyzer for determination of radioactivity (B). The proteins on the other gel were transferred to a polyvinylidenedifluoride membrane (Millipore). The membrane was probed with S-proteinalkaline phosphatase conjugate (Novagen) and the colour was developed according to the manufacturer (C). The positions of ZmCDPK9 fusion protein and casein in the gel are indicated by dots and arrowheads, respectively. The positions and sizes of molecular weight markers (A and B, Sigma; and C, Novagen) are presented in kDa on the left.

pETPK9 were grown in LB medium containing 50 μg/ml ampicillin at 37°C. An overnight culture of *E. coli* was diluted 10-fold and grown for an additional 3 h at 37°C before addition of 1.0 mM (in final concentration) isopropyl-1-thio-β-galactopyranoside and then it was grown for an additional 25 h at 25°C. Extraction of the enzyme was performed essentially as described [14,15]. The protein concentration was determined by the method of Bradford [16]. The reaction mixture (final volume, 20 μl) comprised 20 μM [γ-^{32}P]ATP (4 μCi), 50 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 20 μg casein (Sigma), and the enzyme (20 μg protein). It was incubated at 25°C for 30 min, and separated by 10% SDS-PAGE, and then the radioactivity was determined with a Bio-imaging analyzer (Fuji).

As shown in Fig. 4B, a cell-free extract of *E. coli* containing the recombinant chimeric protein efficiently phosphorylated casein, an exogenous substrate. The expression of fusion protein was confirmed by Western blotting (Fig. 4C). Notably, the enzyme activity was completely inhibited by an excess amount of EGTA. It is also noteworthy that the mobility of ZmCDPK fusion protein in the SDS-polyacrylamide gel was altered by the presence of EGTA (Fig. 4A and C). This Ca^{2+}-dependent mobility shift, generally regarded as a characteristic of high-affinity Ca^{2+}-binding proteins, indicated that the ZmCDPK9 protein binds Ca^{2+} [17,18]. Thus, it was proved that ZmCDPK9 encodes a typical CDPK. The upper bands in Fig. 4B might result from autophosphorylation of the expressed fusion protein. Phosphoenolpyruvate carboxylase from maize was a poor substrate for this CDPK (data not shown). It is important to clarify the physiological role of the protein kinase in the future.

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EXHIBIT B

The Plant Journal (2000) 23(3), 319–327

Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants

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Summary

A rice gene encoding a calcium-dependent protein kinase (CDPK), OsCDPK7, was induced by cold and salt stresses. To elucidate the physiological function of OsCDPK7, we generated transgenic rice plants with altered levels of the protein. The extent of tolerance to cold and salt/drought stresses of these plants correlated well with the level of OsCDPK7 expression. Therefore, OsCDPK7 was shown to be a positive regulator commonly involved in the tolerance to both stresses in rice. Over-expression of OsCDPK7 enhanced induction of some stress-responsive genes in response to salinity/drought, but not to cold. Thus, it was suggested that the downstream pathways leading to the cold and salt/drought tolerance are different from each other. It seems likely that at least two distinct pathways commonly use a single CDPK, maintaining the signalling specificity through unknown post-translational regulation mechanisms. These results demonstrate that simple manipulation of CDPK activity has great potential with regard to plant improvement.

Keywords: CDPK, cold, salt, drought, rice, stress tolerance.

Introduction

Environmental stresses, such as cold, salinity and drought, have an enormous impact on crop productivity throughout the world (Boyer, 1982; Epstein *et al.*, 1980). To survive under unfavourable conditions, plants have developed a variety of sophisticated strategies (Bohnert *et al.*, 1995; Bray, 1997; Thomashow, 1999; Zhu *et al.*, 1997). The products of some stress-inducible genes (e.g. enzymes involved in the biosynthesis of various osmoprotectants and late-embryogenesis-abundant (LEA) proteins) directly counteract the detrimental conditions. Transfer of these genes into plants confirmed their protective roles in stress adaptation (Holmberg and Bulow, 1998; Xu *et al.*, 1996). Although the effect of each individual gene is rather small, simultaneous transcriptional activation of a subset of these genes can confer much greater stress tolerance on *Arabidopsis* (Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998). It is generally thought that distinct mechanisms underlie the adaptation to cold and salt/drought stresses in plants (Shinozaki and Yamaguchi-Shinozaki, 1997; Zhu *et al.*, 1997). Both appear to be regulated by complex signalling networks of abscisic acid (ABA)-dependent pathways and/or ABA-independent pathways (Ishitani *et al.*, 1997; Leung and Giraudat, 1998; Shinozaki and Yamaguchi-Shinozaki, 1997). It has been predicted that the modulation of signalling regulators will be a promising method for improving the stress tolerance of plants. However, little is known about the molecular mechanisms underlying signal transduction.

Previous studies showed that the cytoplasmic Ca^{2+} levels in plant cells increase rapidly in response to multiple stress stimuli, including cold, salt and drought (Sanders *et al.*, 1999; Trewavas and Malho, 1997). Following this Ca^{2+} influx, signals are likely to be mediated by combinations of protein phosphorylation/dephosphorylation cascades. The experimental perturbation with specific reagents indicated the pivotal roles of Ca^{2+} influx and protein phosphorylation in these stress responses (Knight *et al.*, 1996; Knight *et al.*, 1997; Monroy and Dhindsa, 1995; Monroy *et al.*, 1993; Monroy *et al.*, 1998; Tahtiharju *et al.*, 1997). It is presumed that the majority of Ca^{2+}-stimulated

protein phosphorylation is performed predominantly by members of the Ca^{2+}-dependent protein kinase (CDPK) family in plants (Sanders *et al.*, 1999; Trewavas and Malho, 1997). This kinase family, currently only known in plants and protozoa, contains a calmodulin-like regulatory domain with four EF hands, Ca^{2+}-binding site, at its C-terminal end, which enables activation directly through Ca^{2+} binding (Roberts and Harmon, 1992). Two of the four constitutively active mutant enzymes of two related *Arabidopsis* CDPKs activated a stress/ABA-responsive promoter in a transient expression system, indicating that selected members of the CDPK family are involved in that particular stress signalling (Sheen, 1996). In addition, stress-induced gene expression of some CDPK members has been reported in various plant species, and they are proposed to mediate stress signals (Berberich and Kusano, 1997; Botella *et al.*, 1996; Urao *et al.*, 1994; Yoon *et al.*, 1999). However, it is not known whether or not the increases in mRNA levels are accompanied by increases in protein levels and/or kinase activities. It is also noteworthy that a number of transport proteins (e.g. aquaporins, H^+-ATPases and ion channels), which are responsible for cytosolic osmoregulation and involved in stress adaptation, are regulated by CDPKs (Bethke and Jones, 1997; Camoni *et al.*, 1998a; Li *et al.*, 1998; Lino *et al.*, 1998; Pei *et al.*, 1996; Weaver and Roberts, 1992). Despite the potential importance of CDPKs, the physiological function of a specific CDPK pathway has not been elucidated so far.

In the current study, we investigated the function of a rice cold- and salt-inducible CDPK, OsCDPK7, by using transgenic rice plants with altered levels of the protein. Over-expression of OsCDPK7 conferred both cold and salt/drought tolerance on rice plants. In contrast, suppression of OsCDPK7 expression lowered the stress tolerance. The results indicate that OsCDPK7 plays key roles in the tolerance to both stresses in rice.

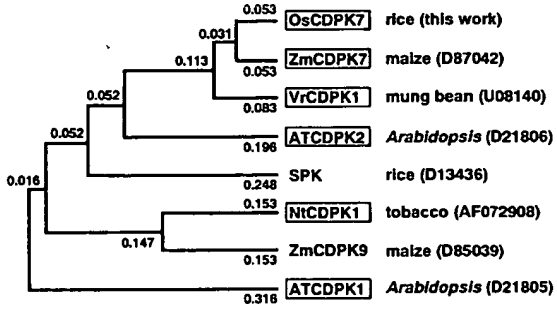

Figure 1. Phylogenetic tree for the amino acid sequences of CDPKs.
The UPGMA method (Nei, 1987) was applied using Genetyx-Mac software (Software Development, Tokyo, Japan). The values on the branches give branch length as the number of amino acid substitutions per site. CDPKs, the transcripts of which are stress-inducible, are boxed. Plant sources and GenBank accession numbers are indicated.

Results

cDNA cloning of OsCDPK7

Two maize CDPKs, ZmCDPK1 and ZmCDPK7, showed 97.1% identity at the amino acid level over the entire polypeptide (Berberich and Kusano, 1997; Saijo *et al.*, 1997), and the mRNA expression of both CDPK genes were induced by cold stress (Berberich and Kusano, 1997; data not shown). Using the *ZmCDPK7* cDNA as a probe, we isolated a highly homologous rice clone, designated *OsCDPK7* (GenBank accession no. AB042550), from a rice root cDNA library (Hata *et al.*, 1997). In contrast to the maize genome, Southern blot analysis of rice genomic DNA with the 3′-non-coding region (nt 1815–2061) as a probe indicated that the rice genome contains only a single copy of this gene (data not shown).

OsCDPK7 and ZmCDPK7 showed 88.6% identity at the amino acid level over the entire polypeptide. In addition, OsCDPK7 showed high amino acid identity to a mung bean CDPK, VrCDPK1 (85.4%), the transcript level of which was elevated by salt stress and mechanical strain (Botella *et al.*, 1996). They were classified into the same subclass of CDPKs in a phylogenetic tree for the amino acid sequences of the entire ORFs (Figure 1). A further database search revealed an *Arabidopsis* expressed sequence tag (EST) clone (accession no. R90026) encoding a portion of CDPK (124 amino acids). The deduced protein sequence was more closely related to OsCDPK7 than ATCDPK1 (Sheen, 1996; Urao *et al.*, 1994), a putative *Arabidopsis* stress-signalling isoform (amino acid identities of 80.6 and 52.8%, respectively). Therefore, it seems likely that OsCDPK7, ZmCDPK7, ZmCDPK1, VrCDPK1 and the *Arabidopsis* protein may be orthologues that play identical roles under stress conditions.

The entire coding region of OsCDPK7 was subcloned into an expression vector, pGEX4T-1 (Amersham Pharmacia), and then transformed into *Eschericia coli* BL21 (DE3). Protein kinase activity was assayed as described (Saijo *et al.*, 1997). The recombinant OsCDPK7 protein fused with glutathione-*S*-transferase (GST) efficiently phophorylated histone IIIS, casein, and myelin basic protein (Sigma) in a Ca^{2+}-dependent manner (data not shown). This broad substrate specificity is in sharp contrast to the narrow specificity of a GST–ATCDPK1 fusion protein (Urao *et al.*, 1994), indicating that OsCDPK7 and ATCDPK1 differ in substrate preferences.

OsCDPK7 *is induced by cold and salt stresses*

Expression of the *OsCDPK7* mRNA, around 2.3 kb in size, was increased by cold and salt stresses in both shoots (Figure 2a) and roots (data not shown) of 10-day-old seedlings, but not by exogenous abscisic acid (ABA) application. In contrast, a rice stress-responsive gene,

rab16A (Mundy and Chua, 1988), was induced by the salt and ABA treatments (Figure 2a). This indicates that OsCDPK7 belongs to a subclass of stress-inducible CDPKs, which is probably conserved from monocotyledonous to dicotyledonous plants.

To determine whether or not the protein level increases upon stressing, we then carried out immunoblot analyses with isoform-specific antibodies raised against the C-terminal portion of OsCDPK7. A clear band corresponding to an apparent molecular mass of 51 kDa was seen in the soluble fractions of the protein extracts from plant tissues (Figure 2b). Significant variation in the protein level was not detected during the cold-stress period examined in either the shoots (Figure 2b) or roots (data not shown), even when the mRNA accumulated to a high level (Figure 2a). The protein levels were almost similar between shoots and roots (data not shown). In the microsomal membrane fractions precipitated by the centrifugation, no signal for the OsCDPK7 protein was detected (data not shown). Similar results were obtained under salt stress (data not shown). Thus, the intracellular localization of the enzyme protein is as yet unclear. Moreover, the shoot proteins immunoprecipitated with anti-OsCDPK7 antibodies did not exhibit any changes in kinase activity (data not shown). However, the activity seemed to be affected by contaminated Ca^{2+} during the preparation of protein extracts. Therefore, in this experiment, additional factor(s) that positively or negatively influence the activity other than Ca^{2+} could not be detected either.

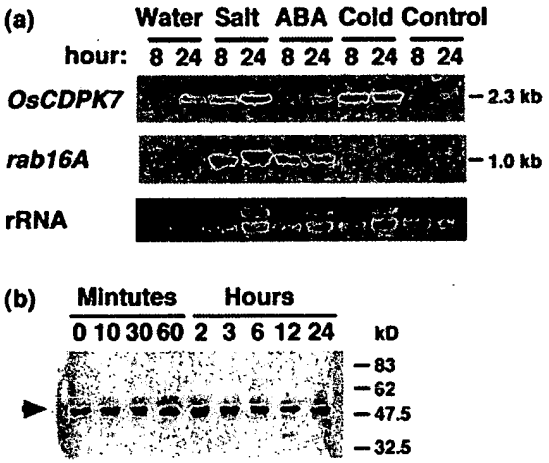

Figure 2. Expression of *OsCDPK7* in wild-type rice plants.
(a) Cold and salt induction of the *OsCDPK7* transcript. Ten-day-old rice (*Oryza sativa* cv. Notohikari) seedlings in soil were left to stand under the normal conditions at 28°C (control) or at 4°C (cold) for the indicated times. Alternatively, they were transferred to nutrient solution comprising 0.1% (v/v) Hyponex (water) supplemented with 200 mM NaCl (salt) or 50 μM ABA (ABA), and then incubated at 28°C for the indicated times. These treatments were conducted under continuous light conditions. Total RNA preparations (10 μg lane^{-1}) from shoots were loaded. The RNA blot was sequentially hybridized with the *OsCDPK7*-and *rab16A*-specific DNA probes.
(b) OsCDPK7 protein levels. Soluble protein extracts were prepared from shoots of 10-day-old seedlings which had been incubated at 4°C for the indicated times. The extracts (10 μg protein lane^{-1}) were separated by 10% SDS–polyacrylamide gel electrophoresis, and then subjected to immunoblot analysis with anti-OsCDPK7 antibodies. An arrowhead indicates the position of OsCDPK7.

Transgenic rice plants with altered expression levels of OsCDPK7

To clarify the physiological role of OsCDPK7 in rice, the full-length cDNA (nt 1–1970) for OsCDPK7 was introduced into rice cells in the sense orientation under the control of the cauliflower mosaic virus (CaMV) 35S promoter (Figure 3a) by means of *Agrobacterium*-mediated transformation (Hiei *et al.*, 1994). Then, 14 independent lines of transgenic plants (T0 generation) were generated. DNA blot analysis of these lines confirmed that independent lines contained one to several copies of the transgene per haploid genome (data not shown). Among them, two lines over-expressing *OsCDPK7*, S3 and S1, and another co-suppressed line, S27, were chosen for further experiments. In the next T1 generation, the levels of the OsCDPK7 protein were constitutively higher in the two over-expressing lines, S3 and S1, than those in the segregated non-transgenic (NT) line derived from S3 in both roots and shoots (Figure 3b). No obvious effects on plant growth and development were observed on OsCDPK7 over-expression under the normal growth conditions. On the other hand, the protein level in S27 was much lower than that in NT (Figure 3b). Since the protein levels were similar between homozygous and heterozygous plants in each line (data

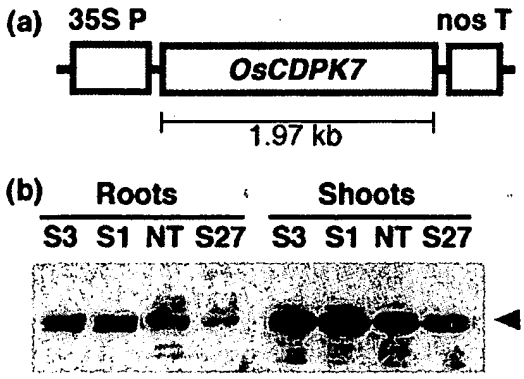

Figure 3. Transgenic rice plants with altered protein levels of OsCDPK7.
(a) The OsCDPK7 expression vector for rice transformation. 35S P, CaMV 35S promoter; *OsCDPK7*, the full-length cDNA (1.97 kb in size) for OsCDPK7; nos T, terminator sequence of the gene for nopaline synthase.
(b) Expression of the OsCDPK7 protein in transgenic and non-transgenic rice plants. NT, a segregated non-transgenic line derived from S3. Each lane contained 5 μg (roots) or 10 μg (shoots) protein from 10-day-old T1 seedlings grown under the normal conditions. The protein blots were probed with anti-OsCDPK7 antibodies. An arrowhead indicates the position of OsCDPK7.

not shown), both types of T1 transformants were combined and then used for further analyses. Significant variations in the protein level in each line were not detected even under cold- and salt-stress conditions (data not shown), when the transcript levels were increased (also see Figure 5).

Stress tolerance of transgenic OsCDPK7 *rice plants*

To examine cold tolerance, 10-day-old T1 seedlings were exposed to 4°C for 24 h, and then returned to the normal growth conditions to allow their recovery. The extent of cold tolerance correlated well with the level of OsCDPK7 expression (Figure 4a). The elevated tolerance of the OsCDPK7-over-expressing plants was confirmed by measuring the changes in the chlorophyll fluorescence yield in the youngest extended leaf of each plant. The F_v/F_m values recovered to nearly normal levels in S3 and S1 plants 48 h after cold treatment (Figure 4b). In contrast, the values progressively decreased in the segregated NT plants derived from S3, their leaves showing prominent chlorosis and wilting. In another independent experiment, similar results were obtained (Table 1).

Next, the over-expressing plants also showed an increased tolerance to salt stress (Figure 4c). In more than half of the NT-S3 plants and untransformed wild-type plants, the youngest leaves wilted 3 days after salt stress. On the other hand, S3 and S1 plants exhibited greater tolerance (statistically significant) (Table 1). In addition, 13-day-old T2 seedlings of S1 plants showed increased drought tolerance with statistical significance (Figure 4d and Table 1). All the NT plants wilted 5 days after drought stress, whereas more than half of the S1 plants did not.

To determine whether or not a decrease in OsCDPK7 expression lowers the stress tolerance, we compared the number of plants whose leaves wilted under milder conditions between the OsCDPK7-suppressed plants (S27) and the segregated NT plants derived from S27. In

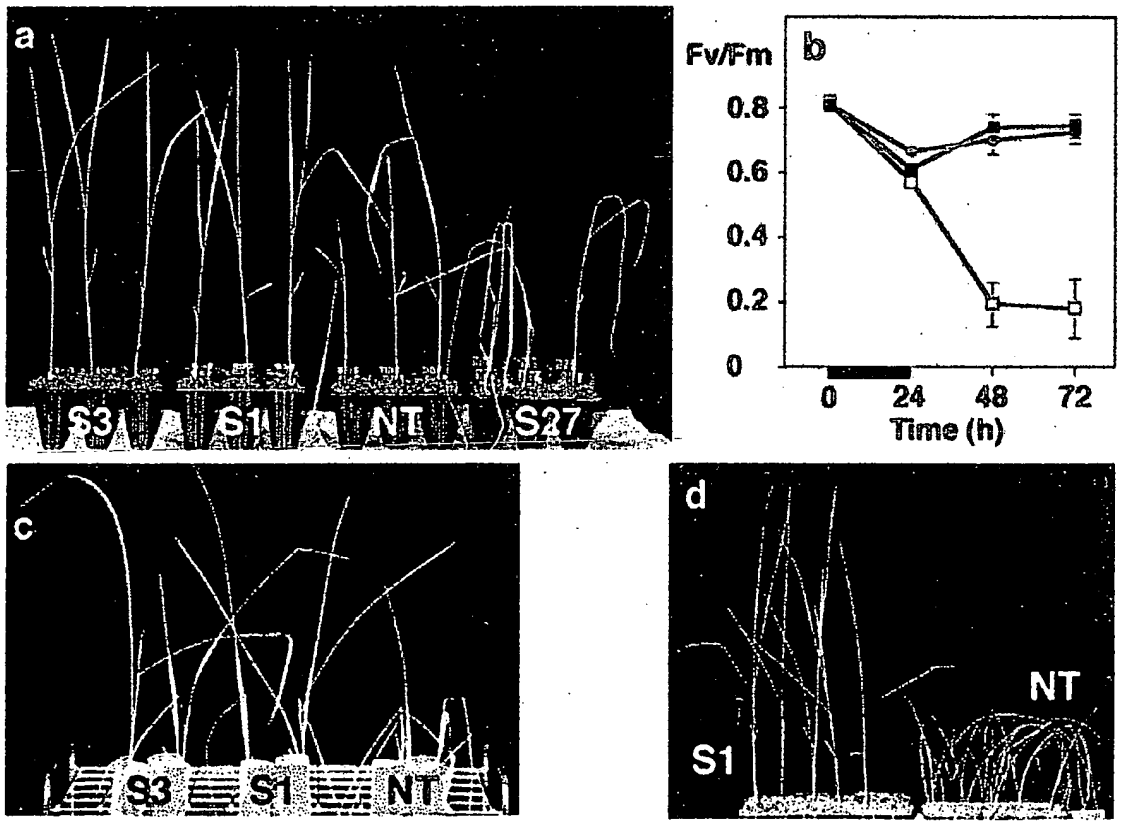

Figure 4. Stress tolerance of 35S:*OsCDPK7* transgenic rice plants.
(a) Plants 3 days after cold stress (4°C for 24 h). (b) Changes in the chlorophyll fluorescence of the youngest extended leaf under cold stress. Each point shows the mean and standard error of F_v/F_m values, from which functional damage to photosynthesis can be estimated. (■) S3; ( ) S1; (□) NT. For the S3, S1, and NT lines, 25, 21 and 9 plants, respectively, were assayed. The bar below the *x* axis represents the period of cold-stress treatment. (c) Plants 3 days after salt stress (200 mM NaCl for 24 h). (d) Plants 5 days after drought stress (without water supply for 3 days).

S27 plants, the tolerance to both of these stresses was significantly decreased as follows. After milder cold stress, wilting was observed in 18/33 (55%) S27 plants and 3/9 (33%) NT plants. After milder salt stress, the wilting ratios of S27 and NT plants were 9/35 (26%) and 0/12 (0%), respectively. Taken together, we conclude that OsCDPK7 is a positive regulator commonly involved in the adaptation to at least three distinct stress agents, cold, salt and drought.

Expression of stress-inducible genes in transgenic OsCDPK7 *plants*

The induction of numerous stress-responsive genes is a hallmark of stress adaptation in plants (Shinozaki and Yamaguchi-Shinozaki, 1997; Thomashow, 1999; Zhu *et al.*, 1997). To elucidate further the role of OsCDPK7 in stress tolerance, we examined the effects of OsCDPK7 over-expression on the transcript levels of several stress-inducible rice genes – *rab16A* (Mundy and Chua, 1988), *salT* (Claes *et al.*, 1990) and *wsi18* (Takahashi *et al.*, 1994) – that encode a group 2 late-embryogenesis-abundant (LEA) protein, a glycine-rich protein, and a group 3 LEA protein, respectively. Since it is thought that similar mechanisms underlie the adaptation to water deficit caused by drought and high salinity (Bray, 1997; Zhu *et al.*, 1997), we analysed the expression of these genes in roots only under cold and salt stresses.

In both roots and shoots of the OsCDPK7-over-expressing plants (S3 and S1), the *OsCDPK7* transcript was accumulated at high concentrations. The level was increased further under both cold and salt stresses, whereas it remained almost constant in the presence of exogeneous ABA or under drought stress conditions (Figure 5). Notably, OsCDPK7 over-expression did not induce the above stress-inducible genes under the normal growth conditions (Figure 5), in contrast to resukts obtained for the *CBF1*-and *DREB1A*-over-expressing *Arabidopsis* plants (Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998). In roots of the over-expressing plants under salt stress, the transcript levels of all the stress-inducible genes were higher than the normally induced levels (Figure 5a). In shoots, only *rab16A* was highly induced by both salt and drought stress (Figure 5b,c). In contrast, salt induction was reduced in the suppressed plants (Figure 5a,b). The salt/drought stress tolerance of the over-expressing plants may be enhanced, at least in part, by the high-level accumulation of these

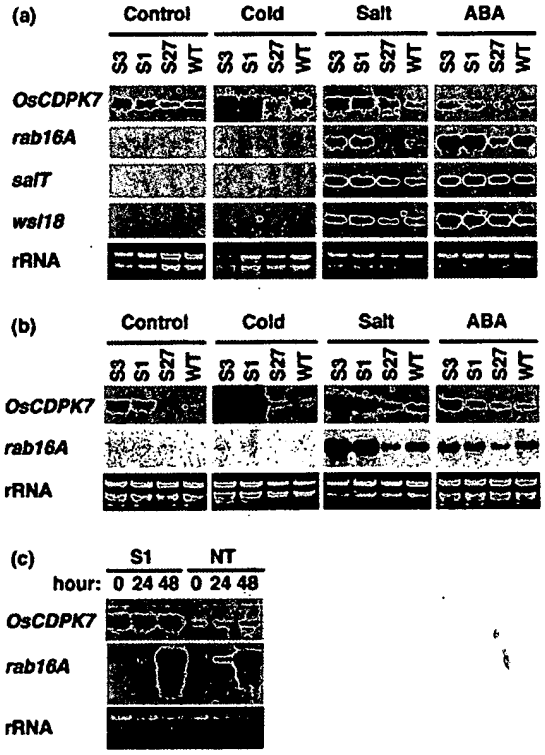

Figure 5. OsCDPK7 activates distinct signalling pathways in response to cold and salt/drought stresses.
(a,b) Northern analyses of RNAs of roots (a) and shoots (b) of wild-type (WT) and transgenic T1 plants. Ten-day-old seedlings were exposed to the following stress conditions. Control, the normal conditions; cold, 4°C for 24 h; salt, soaking in a 200 mM NaCl solution for 10 h (a) or 24 h (b); ABA, soaking in a 50 μM ABA solution for 24 h. (c) Northern analysis on transgenic T2 plants exposed to drought stress. Thirteen-day-old seedlings of the plants homozygous for the transgene (S1) and those of NT plants had water withheld for the indicated times. For each treatment, at least 7 plants per line were examined. Aliquots (10 μg) of total RNA were loaded on each lane. The RNA blots were hybridized with the specific DNA probes for *OsCDPK7*, *rab16A*, *salT* and *wsi18*. Experiments were repeated three times with similar results.

Table 1. Cold, salt and drought stress tolerance of the wild-type (WT) and transgenic *OsCDPK7* rice plants

Stress	Genotype	Total	Wilting (%)
Cold[a]	S3[d]	20	0 (0)
	S1[d]	17	2 (12)
	NT-S3	6	5 (83)
Salt[b]	S3[d]	21	3 (14)
	S1[d]	12	0 (0)
	NT-S3	7	4 (57)
	WT	7	4 (57)
Drought[c]	S1[d]	17	8 (47)
	NT-S1	24	24 (100)

[a]Numbers of WT and T1 plants, the youngest leaves of which wilted 3 days after cold stress (4°C for 24 h). [b]Numbers of WT and T1 plants, the youngest leaves of which wilted 3 days after salt stress (200 mM NaCl for 24 h). [c]Numbers of wilting T2 plants 5 days after drought stress (without water supply for 3 days). [d]Genotypes showing a statistically significant difference from the NT plants (χ^2 test, $P < 0.005$).

gene products through the OsCDPK7 pathway. On the other hand, no induction of these genes was detected under cold stress. Thus, it was suggested that mechanisms of cold tolerance and salt/drought tolerance are different from each other, sharing OsCDPK7 as a common component. Moreover, it should be noted that the induction of *rab16A* and *salT* by ABA was increased or decreased by OsCDPK7 over-expression or suppression, respectively, which indicates that OsCDPK7 is also involved in ABA-dependent pathways.

In addition to the above-mentioned genes, the salt/ABA induction but not cold induction of *rab16B* (Yamaguchi-Shinozaki *et al.*, 1989) and *oslea3* (Moons *et al.*, 1997), both of which encode LEA proteins, was found to be increased in roots of the over-expressing plants (data not shown). The expression of the following rice cold-responsive genes was also examined: *alcohol dehydrogenase-1* (Xie and Wu, 1989), *lip5*, *lip9'*, and *lip19* (Aguan *et al.*, 1991), Δ^1-*pyrroline-5-carboxylate synthetase* (Igarashi *et al.*, 1997) and *glutathione reductase-2* (Kaminaka *et al.*, 1998). However, they exhibited no correlated induction with the OsCDPK7 expression levels (data not shown). Thus, we have not yet identified genes induced only by cold stress. Nevertheless, it still remains possible that currently unknown genes are regulated by the OsCDPK7 pathways under cold stress.

Discussion

Considering its importance as the most major crop in the world, a better understanding of stress signalling in rice would undoubtedly have an enormous impact. Here we characterized a rice CDPK, OsCDPK7, the mRNA level of which is increased under cold- and salt-stress conditions in both shoots and roots. Database searches revealed the presence of genes encoding this type of stress-inducible CDPK in both monocots and dicots. OsCDPK7 belongs to a subclass of stress-inducible CDPKs, which is conserved throughout higher plants but distinct from that of ATCDPK1, a putative stress-signalling isoform (Sheen, 1996; Urao *et al.*, 1994). The difference in substrate specificity also indicated that OsCDPK7 is involved in other signalling pathway(s) than that of ATCDPK1 under stress conditions.

Immunoblot analysis of the protein level of OsCDPK7 in rice plants with the isoform-specific antibodies detected no significant increase under the stress conditions, even when the mRNA level was elevated. There are other examples of putative stress-signalling mitogen-activated protein kinases (MAPKs) in plants in which transcriptional up-regulation is not correlated with an increase in the amount of protein (Bogre *et al.*, 1997; Seo *et al.*, 1999). It may be speculated that the activated MAPK is degraded immediately after it has transduced signal(s), and the transcriptional up-regulation of the MAPK gene is to compensate for the loss of the MAPK protein (Hirt, 1999). To know whether or not this is the case for OsCDPK7, it is important to examine turnover of the protein under the stress conditions. Since the OsCDPK7 protein is expressed at an almost constant level in the presence or absence of stress stimuli, there must be a post-translational mechanism(s) regulating the kinase activity in plant cells. At present, Ca^{2+} is the only known regulator of the activity of CDPKs. However, it seems likely that additional unknown mechanism(s) may be involved in control of the OsCDPK7 activity under stress conditions, taking into account the broad substrate specificity of this kinase. In this regard, recent studies suggested that some of the isoforms might be regulated by interaction with other proteins, e.g. 14-3-3 proteins (Camoni *et al.*, 1998b; Moorhead *et al.*, 1999). Even though neither activation of the OsCDPK7 enzyme upon stress nor protein–protein interaction was detected, the possibility that such regulation mechanisms are involved *in vivo* cannot be ruled out at present. Moreover, it is still possible that changes in protein localization could be important in the regulation of OsCDPK7.

We then carried out a functional analysis of the role of OsCDPK7 in stress tolerance of rice plants, using transgenic plants with altered levels of the OsCDPK7 protein. The mRNA derived from the endogenous promoter and that derived from the CaMV 35S promoter might differ in their stability, because the transgene is truncated in the 3'-non-coding region. However, it seems likely that the same intact protein is translated from both types of the mRNA, since Western blot analyses detected a single band in each lane of the over-expressing plants (Figure 3b). Over-expression of OsCDPK7 conferred both cold and salt/drought tolerance on rice plants. In contrast, suppression of OsCDPK7 expression lowered the stress tolerance. Therefore, OsCDPK7 plays key roles in the tolerance to the two types of stress in rice. To our knowledge, this is the first demonstration of the physiological role of a CDPK isoform at the whole-plant level.

The enhanced salt/drought induction of the genes for LEA proteins by OsCDPK7 over-expression appeared to contribute, at least in part, to the improved salt/drought tolerance in rice plants. Notably, the over-expression of a barley (*Hordeum vulgare* L.) group 3 LEA protein, HVA1, conferred both salt and drought stress tolerance on transgenic rice plants (Xu *et al.*, 1996). Although OsCDPK7 over-expression also elevated the ABA-induced levels of the above genes, whether or not the salt induction of these genes depends on ABA remains to be examined.

In contrast to salt/drought stress, no induction of these genes was observed under cold stress or in the absence of a stress stimulus. Although little is known about the OsCDPK7-mediated cold signalling pathway, we suggest that OsCDPK7 promotes cold and salt/drought tolerance

through distinct pathways. Moreover, it seems likely that OsCDPK7 is kept normally inactive, since there is no constitutive induction of the above stress-inducible genes upon OsCDPK7 over-expression. Thus, OsCDPK7 over-production only is insufficient to trigger the downstream signalling, and stress stimuli must be required to activate this CDPK. Consistent with this speculation, no significant effects were observed with regard to development, growth and fertility in over-expressing plants grown in a greenhouse (unpublished results). This is very favourable for crop improvement. For instance, *DREB1A*-over-expressing plants showed severe growth retardation under normal growth conditions, presumably because of the constitutive high-level expression of stress-inducible genes (Liu *et al.*, 1998). In contrast to this transcription factor, it appears that the activity of OsCDPK7 is under a stringent post-translational control in rice cells.

Finally, we propose a model for the OsCDPK7 signalling pathway under the above stress conditions, in which the amount of activated OsCDPK7 determines the transduction current (Figure 6). The results also suggest that OsCDPK7 acts at one of the branch points of stress signal transduction in rice. Nevertheless, there seems little or no cross-talk downstream of these CDPK pathways even when each signal is amplified by OsCDPK7 over-expression. There must be unknown mechanisms that maintain the signalling specificity. Future analyses, especially on protein–protein interactions and protein localization, are awaited to verify this model. The present work provides new approaches for engineering of crop plants with improved stress tolerance, as well as for understanding the principles governing CDPK-mediated stress signal transduction.

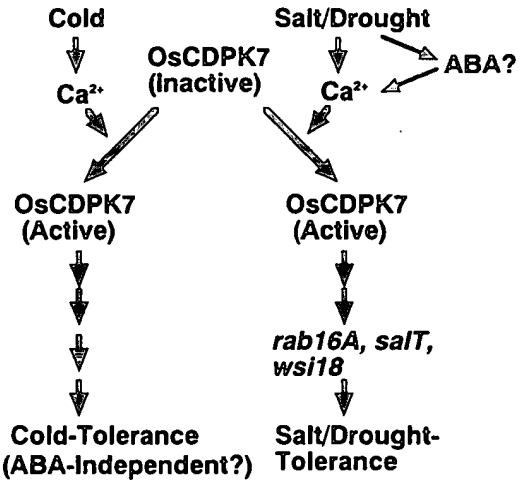

Figure 6. A model for OsCDPK7-mediated cold-and salt/drought-stress signal transduction.

Experimental procedures

cDNA cloning

Using a cDNA for ZmCDPK7 as a probe, cDNA cloning of *OsCDPK7* was carried out as described by Saijo *et al.* (1997) from a rice (*Oryza sativa* Nipponbare) root cDNA library (Hata *et al.*, 1997). Two positive clones were isolated, and both of them were confirmed to encode OsCDPK7 by sequencing. Clones 1 and 2 correspond to nt 1–1970 and nt 453–2126, respectively, of the composite cDNA sequence for OsCDPK7.

Northern blot analysis

Total RNA was prepared from plant tissues according to Clark (1997). Northern blot analysis was performed as described by Saijo *et al.* (1997) using the following probes: the 3′-non-coding region of *OsCDPK7* (nt 1815–2061), and portions of *rab16A* (nt 669–899) (Mundy and Chua, 1988), *salT* (nt 290–690) (Claes *et al.*, 1990) and *wsi18* (nt 678–933) (Takahashi *et al.*, 1994).

Production of anti-OsCDPK7 antibodies

To prepare anti-OsCDPK7 antibodies, a cDNA fragment encoding the C-terminal portion (amino acid residues 449–551) of OsCDPK7 was subcloned into the pET32a vector (Novagen), and then transformed into *E. coli* BL21 (DE3). The expressed recombinant protein was purified on a histidine tag affinity column (Novagen), and then the peptide tags on the N-terminal side were removed by enterokinase digestion. Polyclonal rabbit antibodies raised against the antigen were purified using a column of Sepharose 4B (Amersham-Pharmacia) coupled with the GST–OsCDPK7 fusion protein. The purified antibodies did not cross-react with another CDPK isoform, ZmCDPK9, extracted from *E. coli* (BL21) cells harbouring pETPK9 (Saijo *et al.*, 1997).

Preparation of protein extracts and immunoblot analysis

Plant tissues were ground in liquid nitrogen and then homogenized in an extraction buffer (50 mM Tris–HCl (pH 7.6), 2 mM EDTA, 1 mM $MgCl_2$, 2 mM DTT, 1 mM NaF, 10 mM β-glycerophosphate, 0.1 mM Na_3VO_4, 1 mM phenylmethanesulphonyl fluoride, one tablet per 50 ml protease inhibitor cocktail (Boehringer), 10% (v/v) polyvinylpolypyrrolidone). The homogenate was centrifuged first at 10 000 *g* for 10 min, and then at 100 000 *g* for 45 min. The supernatant was transferred into clean tubes, immediately frozen in liquid nitrogen, and then stored at −80°C. For immunoblot analysis, the supernatant was desalted by passing it through a Sephadex G-25 column (Amersham-Pharmacia) equilibrated with 50 mM Tris–HCl (pH 7.6), containing 1 mM DTT. Western blot analysis was performed essentially as described by Ueno *et al.* (2000) with anti-OsCDPK7 antibodies. The signals were then detected by an enhanced chemiluminescence system (Boehringer).

Rice transformation

A full-length cDNA clone (nt. 1–1970) for OsCDPK7 was introduced into a Ti-based vector, pMSH1 (Kawasaki *et al.*, 1999), in the sense orientation downstream of the CaMV 35S promoter. The construct was introduced into rice calli (*Oryza sativa* cultivar Notohikari) by means of *Agrobacterium*-mediated transformation, according to the published protocol (Hiei *et al.*, 1994).

Transformed calli were selected for hygromycin resistance, and then transgenic plants were regenerated.

Estimation of stress tolerance of transgenic rice plants

In a growth chamber (16 h light/8 h darkness, 28°C), sterilized seeds were germinated on Murashige and Skoog agar medium (Murashige and Skoog, 1962) for 5 days, and then transplanted in soil. For cold-stress treatment, 10-day-old seedlings were exposed to 4°C for 24 h under continuous light conditions, and then returned to the normal growth conditions. Stress treatments under milder conditions were conducted as follows. Ten-day-old seedlings were first incubated at 15°C for 24 h, then at 4°C for 24 h, and finally returned to the normal conditions. For salt-stress treatment, 10-day-old seedlings were transferred to a nutrient solution, 0.1% (v/v) Hyponex (Hyponex Japan), containing 200 mM NaCl or 150 mM NaCl for the milder treatments, and then incubated for 24 h under the normal light/dark cycle at 28°C. Immediately after the salt stress, the roots of the plants were rinsed with water, and then hydroponically grown in a fresh nutrient solution without NaCl. Finally, in order to determine whether or not each T1 plant has the transgene, we amplified a portion of *OsCDPK7* (nt 661–945) from the genomic DNA of each plant by means of PCR, using 5′-ACATCGTCATGGAGCTCTGCGCC-3′ and 5′GAGCTACGTAATATGGGCTTCCG-3′ as primers. A 285 bp fragment without intron sequences was amplified from each transgenic plant. The plants of each T1 line examined included both the homozygous and heterozygous plants. For drought-stress treatment, 13-day-old T2 seedlings of the plants homozygous for the transgene (S1) and those of non-transgenic plants (NT) had water withheld for 3 days, and were then irrigated normally for 5 days.

Determination of chlorophyll fluorescence

Measurement of chlorophyll fluorescence was performed with a pulse-amplitude modulation fluorometer (PAM-2000; Walz, Effeltrich, Germany). Fluorescence signals from the youngest extended leaf of each rice plant, which had been dark-adapted for 15 min, were measured at the indicated times. The ratio of F_v to F_m (F_v/F_m) representing the activity of photosystem II was used to assess the functional damage to the plants (Genty *et al.*, 1989).

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